IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: :

Ioannis Moutsatosos et al. : Group Art Unit: 1636 #22

Serial No.: 09/148,234 : Examiner: W. Sandals

Filed: September 4, 1998 :

For: GENETICALLY ENGINEERED CELLS WHICH EXPRESS BONE MORPHOGENETIC PROTEINS :

CERTIFICATE OF MAILING

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DECLARATION UNDER 37 C.F.R. 1.132

Commissioner for Patents
Washington, DC 20231

Sir:

I, DEBRA PITTMAN, hereby declare as follows:

1. That I hold a M.S. degree in Biology;

2. I have been an employee of Genetics Institute for approximately 18 years;

3. I am a researcher with about 22 years of experience in the area of eukaryotic cellular and molecular biology, and I am a co-author on thirty scientific publications (see Exhibit A for Curriculum Vitae);

4. I have studied the effects of Bone Morphogenic Protein 2 (BMP-2) and Bone Morphogenic Protein 10 (BMP-10) in laboratory mice. The experiments were carried out as follows:

Virus Generation: Replication-defective, E1 and E3 deleted, type 5 (del327) recombinant adenovirus was generated by homologous recombination in human embryonic kidney 293 cells (ATCC, Manassas, VA). Virus was generated by cotransfection of BMP-encoding expression plasmids encoding human BMP-2 or human BMP-10, and 9 to 36 map units of adenovirus backbone. Control virus stocks were generated using plasmids containing marker genes [soluble alkaline phosphatase (SEAP), or β-galactosidase (β-gal)]. Recombinant adenovirus was amplified and released from 293 cells by three cycles of freeze thawing. The virus was further purified by centrifugation through two cesium chloride gradients and dialyzed to equilibrium against phosphate buffered saline, pH 7.2 at 4°C. Following dialysis, glycerol was added to a final concentration of 10%, and the virus was stored at -80°C until use. Virus concentration, expressed in particles/ml, was determined by measuring the optical density at 260 nm. Endotoxin levels were measured with the use of a Limulus Amebocyte Lysate kit (BioWhittaker, Walkersville, MD). The virus was further characterized by PCR amplification of the insert using vector specific primers:

Forward Primer: 5'-TGGATGTTGCCTTTACTTCTA-3'
Reverse Primer: 5'TTCACTGCATTCTAGTTGTG-3'

Study 1: BMP-2

In this study C57BL/6J (Jackson Laboratory, Bar Harbor, ME) mice received an intravenous injection of adenovirus encoding BMP-2 or control virus. Analysis included gross pathology, serum chemistries, cell chemistries, and histopathology.

A single dose of 5 X 10^{10} particles of recombinant adenovirus encoding hBMP-2, or beta galactosidase (β-gal, control) was injected intravenously into female C57BL/6J mice, age 7-8 weeks. Animals were analyzed on day 7 and 14 post injection. Groups consisted of four animals each. Animals were anesthetized and blood samples were collected via retro-orbital sinus for differential blood count smears and hematological analysis. Serum was collected via intracardiac bleeds for serum chemistry analysis. Anilytics, Inc. (Gaithersburg, MD) performed cell and serum chemistry analysis. Tissue was harvested, fixed in formalin, and stained with hematoxylin and eosin for histopathology. Collection included the site of injection. Pathology Associates International (Frederick, MD) performed the histopathology studies.

There were no specific clinical chemistry changes, terminal body weight changes, or gross pathology changes.

There were non-specific in liver weights in the BMP-2 and β-gal groups. These increased liver weights were associated with hepatocytic single cell necrosis and mononuclear cell infiltration as non-specific changes induced by the IV administration of the adenoviral vector and heterologous protein expression. There were also non-specific increases in spleen weights in the BMP-2 and β-gal groups. These weight increases

correlated with non-specific lymphoid hyperplasia and/or increased extramedullary hematopoiesis in the spleen.

All other histologic changes were either incidental or non-specific. Typical non-specific lesions of splenic lymphoid hyperplasia, increased splenic extramedullary hematopoiesis, and hepatic single cell necrosis and inflammation are all typical of the background changes expected with the adenoviral vector delivery system. No specific effects of BMP-2 were noted in any tissue.

Study 2: BMP-2

In this study C57BL/6J (Jackson Laboratory, Bar Harbor, ME) mice received a subcutaneous injection of adenovirus encoding BMP-2 or control virus. Analysis included gross pathology, serum chemistries, cell chemistries, and histopathology.

A single dose of 5 X 10^{10} particles of recombinant adenovirus encoding hBMP-2, or beta galactosidase (control) were injected subcutaneously into female C57BL/6J mice, age 7-8 weeks. Animals were analyzed on day 7 and 14 post injection. Groups consisted of four animals each. Animals were anesthetized and blood samples were collected via retro-orbital sinus for differential blood count smears and hematological analysis. Serum was collected via intracardiac bleeds for serum chemistry analysis. Anilytics, Inc. (Gaithersburg, MD) performed cell and serum chemistry analysis. Tissue was harvested, fixed in formalin, and stained with hematoxylin and eosin for histopathology. Collection included the site of injection. Pathology Associates International (Frederick, MD) performed the histopathology.

There were no specific clinical chemistry changes, terminal body weight changes, or gross pathology changes.

There was inflammation at the injection sites of many animals from all groups on both Day 7 and 17 sacrifices. The inflammation was always slight in the buffer control group and ranged from slight to mild in the treatment groups. This inflammation at subcutaneous injection sites is suggestive of a genuine but non-specific immuno-inflammatory response to the adenoviral vector and/or heterologous protein product in the treatment groups.

There were no specific gene expression-related effects observed in this study. There were slight to mild immuno-inflammatory reactions at the subcutaneous injection sites in each of the treatment groups and are most likely due to the virus and not the protein. No specific changes were observed in the animals that received Ad-BMP-2.

Study 3: BMP-10

In this study C57BL/6J (Jackson Laboratory, Bar Harbor, ME) mice received an intravenous injection of adenovirus encoding BMP-10 or control virus. Analysis included gross pathology, serum chemistries, cell chemistries, and histopathology.

A single dose of 5 X 10^{10} particles of recombinant BMP-10 adenovirus was injected intravenously into female C57BL/6J mice, age 7-8 weeks. The control groups in this study were adenovirus β-galactosidase and buffer. Animals were scheduled to be analyzed on day 7 and 14 post injection; however, due to pronounced debilitation and emaciation, all animals were analyzed on day 7. Groups consisted of four animals each. Animals were anesthetized and blood samples were collected via retro-orbital sinus for differential blood count smears and hematological analysis. Serum was collected via intracardiac bleeds for serum chemistry analysis. Anilytics, Inc. (Gaithersburg, MD) performed cell and serum chemistry analysis. Tissue was harvested, fixed in formalin, and stained with hematoxylin and eosin for histopathology. Collection included the site of injection. Pathology Associates International (Frederick, MD) performed the histopathology.

Most changes considered to be associated with gene expression were characteristic of pronounced debilitation. These changes included the gross observations of emaciation, and atrophy of the thymus, the spleen, and occasionally the liver, the decrease in terminal body weight and in absolute and/or relative (to body) liver, spleen and thymus weights. The microscopic correlates were atrophy of the abdominal and subcutaneous fat and atrophy of the thymus. Further microscopic indications of debilitation were found in the lymph node (lymphoid depletion) and stomach (atrophy of the glandular mucosa with single cell necrosis). Congestion was found in several organs (abdominal and subcutaneous fat, femur, and intestine) and correlated to the discolorations observed in these organs at the gross level.

In all animals there was minimal to moderate multifocal liver necrosis. This change was random in distribution and characterized by small areas of coagulation necrosis, approximately the size of one-fourth to two thirds of a lobule. Some of the nectrotic areas were composed of swollen hydropic nectrotic hepatocytes associated with mild influx of granulocytes (vacuolar degeneration and necrosis). These areas were usually sharply demarcated. The pathophysiology of this necrotic phenomenon was not apparent. This change is not typically associated with debilitation in mice. The consistent occurrence of this change in all animals from the BMP-10 group strongly suggests an association with BMP-10 gene expression.

There was a statistically significant and dramatic increase in aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) on day 7 and 14 compared to the β-galactosidase group. All other hematology and clinical chemistry data variations were considered to be of no relevance to gene expression as they were of minimal amplitude, were lacking corresponding microscopic changes, and/or were

not consistent over time. Other microscopic hepatic changes were consistent with lesions associated with the viral system and were associated with a characteristic serum chemistry profile.

Changes considered to be related to test gene expression were found in the BMP-10 group and consisted of pronounced debilitation and emaciation in all animals from sub-groups 1 and 2. This change was responsible for the premature sacrifice of all sub-group 2 animals on day 7. In addition, there was multifocal random hydropic degeneration and coagulation necrosis in the liver in all BMP-10 animals. This microscopic liver change was considered to be related to BMP-10 gene expression. Congestion was found in several organs (abdominal and subcutaneous fat, femur, and intestine) and correlated to the discolorations observed in these organs at the gross level. The significance of the congestion is uncertain but could be associated with the catabolic processes occurring in the fat-storing compartments.

Study 4: BMP-10

In this study C57BL/6J (Jackson Laboratory, Bar Harbor, ME) mice received a subcutaneous injection of adenovirus encoding BMP-10 or control virus. Analysis included gross pathology, serum chemistries, cell chemistries, and hisotpathology.

A single dose of 5 X 10^{10} particles of recombinant adenovirus encoding hBMP-10 or beta galactosidase (control) was injected subcutaneously into C57BL/6J mice, age 7-8 weeks. Animals were analyzed on day 7 and 14 post injection. Groups consisted of four animals each. Animals were anesthetized and blood samples were collected via retro-orbital sinus for differential blood count smears and hematological analysis. Serum was collected via intracardiac bleeds for cell chemistry analysis. Anilytics, Inc (Gaithersburg, MD) performed cell and serum chemistry analysis. Tissue was harvested, fixed in formalin, and stained with hematoxylin and eosin for histopathology. Collection included the site of injection. Pathology Associates International (Frederick, MD) performed the histopathology.

Changes were seen in the injection sites of all 8 animals within this group, both macroscopially and microscopically. Microscopic changes included inflammation, muscle necrosis and/or regeneration, mesenchymal hyperplasia, and focal chondroplasia in one animal. The mesenchymal hyperplasia consisted of proliferation of plump mesenchymal cells along pre-existing tissue planes. The focus of chondroplasia was embedded in the skeletal muscle underlying the subcutis. It consisted of chondrocytes either embedded in a disorganized chondroid matrix, or forming nodular proliferations of hyaline cartilage, admixed with mineralized matrix. There was a thin layer of fibrous tissue surrounding the chondroid nodules that blended with chondroid type cells and plump mesenchymal cells that radiate outward into the surrounding skeletal muscle. The invading cells were associated with muscle degeneration, necrosis and regeneration.

Significant reduction in thymic weight was apparent in the day 14 group, with thymic atrophy noted macroscopically in 2 animals at day 14. This change was not readily apparent microscopically. No other significant changes were noted in organ weight, hematology findings, or clinical chemistry findings.

BMP-10 gene-related tissue responses were present at the injection sites at day 7 and 14. The major responses included subacute inflammation, mesenchymal hyperplasia, and scattered areas of skeletal muscle necrosis and regeneration. In one animal, a focal chondroid nodule was present within a region of mesenchymal hyperplasia at day 14. A significant thymic weight reduction was apparent at day 14. However, morphologic alterations consistent with thymic atrophy were not present. There were no other significant changes in organ weights or clinical pathology related to expression of BMP-10.

Study 5: BMP-10 and BMP-2

In this study C3H/HeJ (Jackson Laboratory, Bar Harbor, ME) mice received a subcutaneous injection of adenovirus encoding BMP-2 or BMP-10. Analysis included gross pathology, serum chemistries, cell chemistries, and histopathology.

A single dose of 5 X 10^{10} particles of recombinant adenovirus was injected subcutaneously into C3H/HeJ mice, age 7-8 weeks. Animals were analyzed on day 7 and 14 post injection. Groups consisted of four animals each. Experimental animals received adenovirus encoding human BMP-10 or human BMP-2. Control animals received adenovirus which encoded secreted alkaline phosphatase (SEAP). Animals were anesthetized and blood samples were collected via retro-orbital sinus for differential blood count smears and hematological analysis. Serum was collected via intracardiac bleeds for serum chemistry analysis. Anilytics, Inc. (Gaithersburg, MD) performed cell and serum chemistry analysis. Tissue was harvested, fixed in formalin, and stained with hematoxylin and eosin for histopathology. Collection included the site of injection. Pathology Associates International (Frederick, MD) performed the histopathology.

BMP-10: The organ weight, biochemical, hematological, and histopathologic profiles were similar to those of the SEAP control group. All hematology and clinical chemistry data variations were considered to be of no relevance to gene expression as they were of minimal amplitude, were lacking corresponding microscopic changes, and were not consistent over time.

The only change of note was found in animals that received adenovirus encoding BMP-10. These changes were at the injection site and consisted of mineralization of the cutaneous muscle in 2/4 Day 7 and 2/4 Day 14 animals. This change was associated with localized degeneration and slight regeneration of skeletal muscle fibers and mild fibro-histiocytic proliferation in the surrounding connective tissue. These changes were of comparable nature and severity in the Day 7 and Day 14 animals.

Changes specific to BMP-10 expression were seen in 2/4 Day 7 and 2/4 Day 14 animals were mineralization of the cutaneous muscle at the site of injection. This was associated with localized degeneration and slight regeneration of skeletal muscle fibers and mild fibro-histiocytic proliferation in the surrounding connective tissue.

5. Based on the experiments described above, I have concluded that BMP-10 and BMP-2 are not functionally equivalent.

6. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

10-22-01
Date

Debra D. Pittman
Debra D. Pittman

GREENLEE, WINNER AND SULLIVAN, P.C.
5370 Manhattan Circle, Suite 201
Boulder, CO 80303
Telephone (303) 499-8080
Facsimile: (303) 499-8089
Email: winner@greenwin.com

Attorney docket No. 47-01
bmk:

Curriculum Vitae
Debra D. Pittman

PERSONAL INFORMATION:

Date of Birth: August 21, 1954
Denver, CO

HOME ADDRESS:

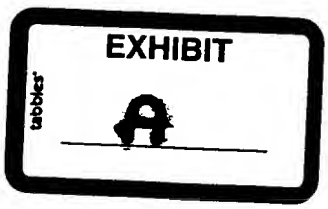
20 North Shore Road
P.O.Box 171
Windham, NH
Phone: (603)898-8929

BUSINESS ADDRESS:

Genetics Institute, Inc
87 Cambridge Park Drive
Cambridge, MA 02140
Phone: (617)876-1170

EDUCATIONAL AND PROFESSIONAL RECORD:

1993-Present	Principal Scientist, Genetics Institute
1989-1993	Staff Scientist II, Genetics Institute
1987-1989	Staff Scientist I, Genetics Institute
1985-1987	Scientist, Genetics Institute
1984-1985	Associate Scientist, Genetics Institute
1983-1984	Assistant Scientist, Genetics Institute
1979-1983	M.S. in Biology, Tufts University, Medford, MA
1978-1979	Special Graduate Student, Stanford, Stanford, CA
1977-1979	Chemist II, SYVA, Palo Alto, CA
1976-1977	Chemist I, SYVA, Palo Alto, CA
1977	B.A. in Biology, University of California, Santa Cruz, CA



1976	Laboratory Technician, SYVA, Palo Alto, CA
1975	Undergraduate Research Assistant, University of California, Santa Cruz, CA
1974-1975	Laboratory Assistant, NASA-AMES Research, Mountain View, CA

Teaching:

Teaching Assistant for Biochemistry, Tufts University, 1980.
Teaching Assistant for Biology for Non-Science Majors, Tufts University, 1980.
Laboratory Instructor for Introductory Biology, Tufts University, 1979.

AFFILIATIONS

American Heart Association
International Society on Thrombosis and Haemostasis

AWARDS AND HONORS:

Young Investigator Award
International Society of Thrombosis and Hemostasis
Tokyo
Japan, 1989

Invited speaker 1990 Congress of the World Federation of Hemophilia (Structure-Function of Factor VIII)
Washington, DC
August, 1990

Chairperson
International Congress on Thrombosis and Haemostasis
Factor V
Amsterdam, Netherlands
July, 1991

PATENTS:

5 U.S. patent applications (2 issued)

PUBLICATIONS:

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